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14. ABSTRACT

This predoctoral training grant, supported by the Department of Defense Breast Cancer Research Program (BCRP) of the Office of the Congressionally Directed Medical Research Programs (CDMRP), aims to study the possible role of membrane transporters in pleitropic drug resistance, using a combination of molecular biological, biochemical, and biophysical methods. The third year of funding was spent in further characterization of the Plasmodium falciparum Chloroquine Transporter (PfCRT), a member of a drug/metabolite transporter superfamily. Data from the previous year provided proof of the protein's direct interaction with its drug substrate. I have subsequently employed a methodology commonly applied to drug resistance proteins (such as human P-glycoprotein) and defined the drug binding site through mass spectrometry. This report summarizes work conducted from March 2008 through June 2008, when my work on this project ceased due to graduation. A request for a change of PI for this award has been submitted and is pending review.

15. SUBJECT TERMS
Drug resistance, drug transporters, photoaffinity labeling, mass spectrometry

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INTRODUCTION

Human P-glycoprotein (Pgp) is a transmembrane glycoprotein implicated in contributing to the resistance of cancer cell lines to chemotherapeutic drugs. The most popular model for the action of Pgp is active drug efflux. Pgp is thought to bind to polar drugs that have passively diffused through the lipid bilayer of the membrane into the cel l and actively pump them out, even against a concentration gradient [1]. An alternative hypothesis accounting for the decreased accumulation of drugs in multidrug resistant (MDR) cells is that the altered partitioning of the drugs between the extracellular and intracellular compartments is due to a perturbation in the chemical environment and/or the number of drug binding sites [2]. Regardless of the mechanism of action, it is now generally accepted that Pgp alone cannot fully account for the spectrum of drug resistance found clinically [3]. This predoctoral training grant, supported by the Department of Defense Breast Cancer Research Program (BCRP) of the Office of the Congressionally Directed Medical Research Programs (CDMRP), aims to study the possible role of membrane drug transporters in pleitropic drug resistance, using a combination of molecular biological, biochemical, and biophysical methods.

BODY

In my first year of work on this project, I heterologously expressed and characterized the P-glycoprotein homologue, the *Plasmodium falciparum* Multidrug Resistance protein (PfMDR1). I found very little remarkable drug stimulation or inhibition of ATPase activity. The contribution of PfMDR1 to malarial drug resistance seems minimal, at best playing a m odulatory role in the resistance pre-determined by other factors. In the second year of work, I focused on the photoaffinity labeling of the *Plasmodium falciparum* Chloroquine Resistance Transporter (PfCRT) with a novel perfluoro-azido-biotinylated chloroquine (CQ) analog called AzBCQ. My results showed that CQ does in fact directly interact with the protein, and competition studies also suggested a physical interaction with other quinoline drugs (*e.g.* quinine), as well as the chemoreversal agent verapamil.

During my abbreviated third year of work, I further characterized the CQ-PfCRT interaction by defining the drug binding site, as has previously been done for human Pgp [4-7]. As a first step, PfCRT (which is labeled with a polyHis tag) was reacted with AzBCQ and then partially digested with trypsin (Figure 1). The major initial degradation product was ~17 kDa and must be the C-terminus of the protein as it retains the polyHis tag (Figure 2). Further digestion did not allow for resolution of smaller labeled fragments, although a ~6.5 kDa unlabeled and non anti-his reactive fragment was identified on comp anion silver-stained gels.

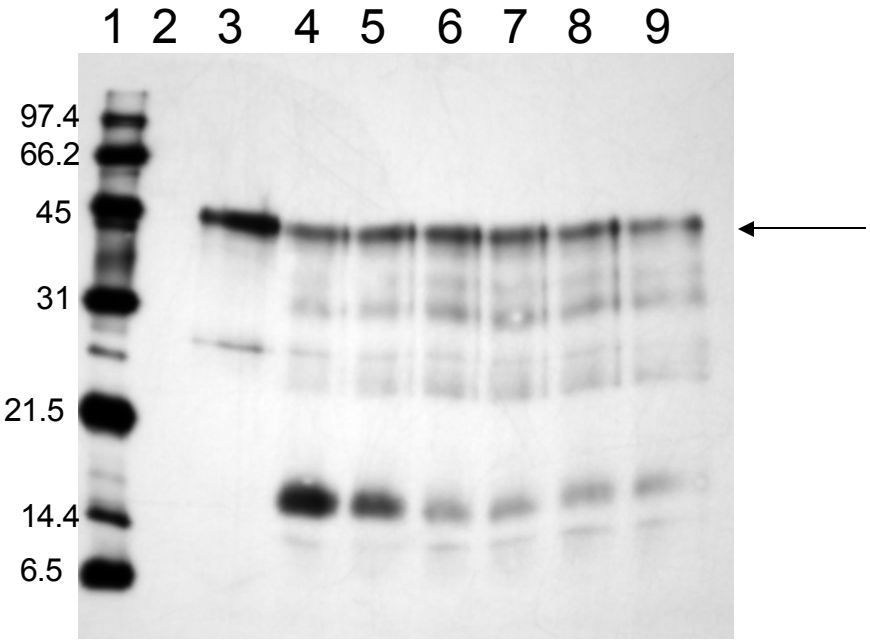
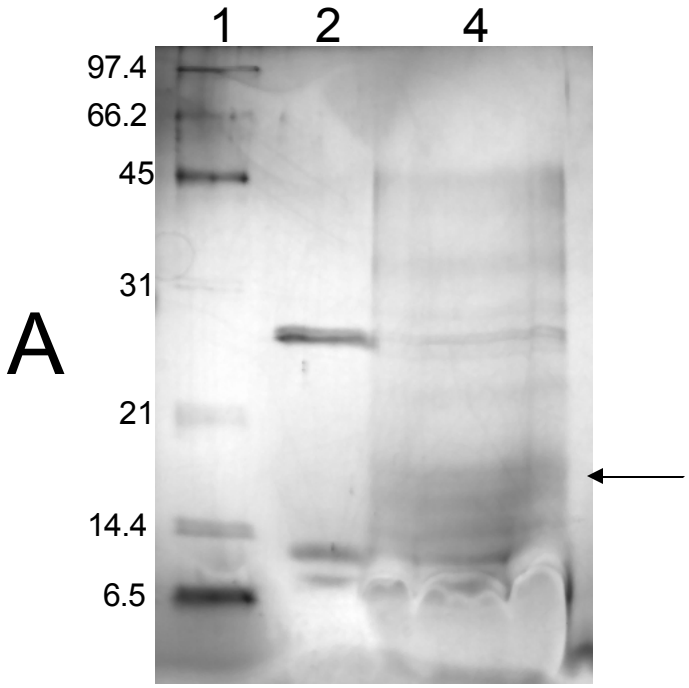


Figure 1 Avidin blot of trypsin digest of AzBCQ -labeled PfCRT. Lane 1, MW standards; lane 2, trypsin enzyme alone; lane 3, undigested PfCRT; lanes 4 -9, PfCRT digested with trypsin for 2.5, 5, 10, 15, 20, and 30 min respectively.



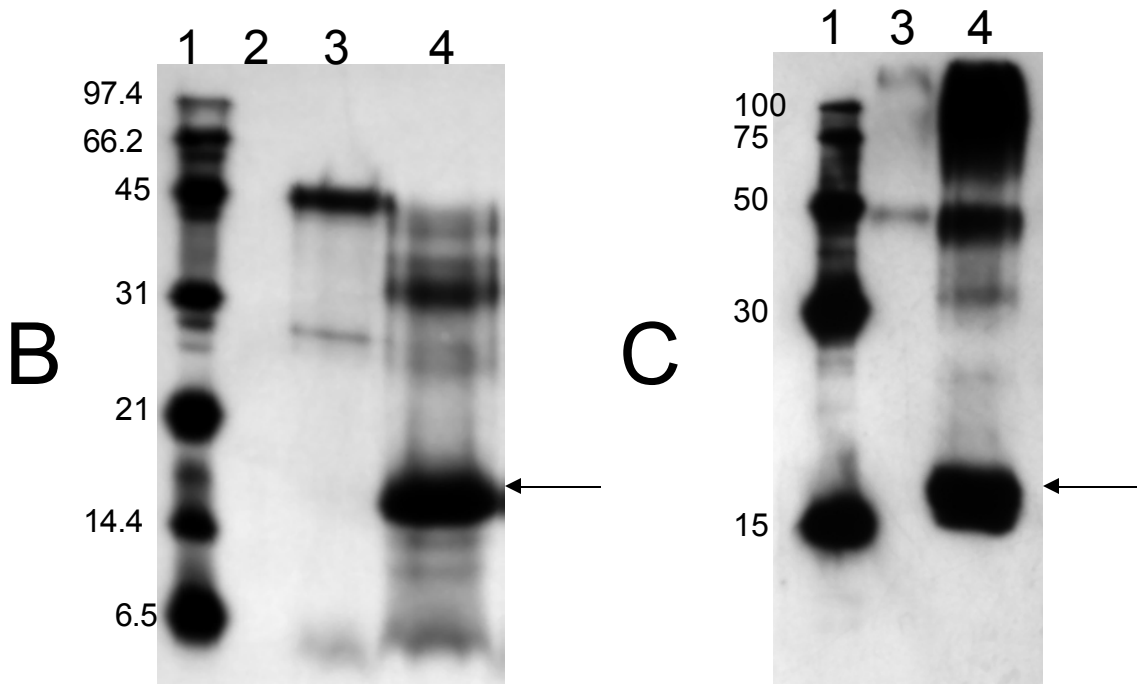
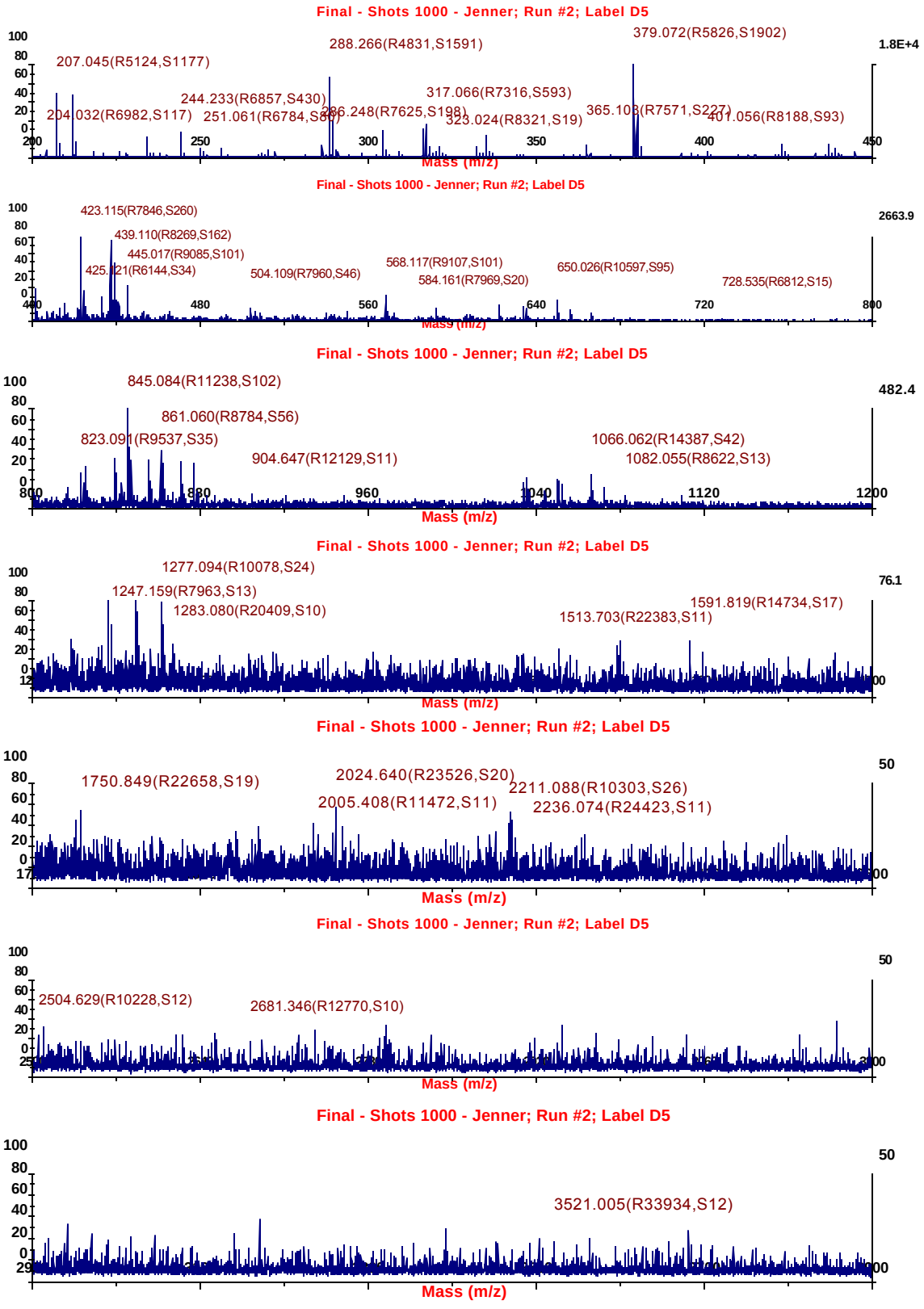


Figure 2 *Identification of the major tryptic fragment as the C-terminus*. (A) silver stain, (B) avidin blot, and (C) polyHis blot of tryptic digest of AzBCQ-labeled PfCRT. Lane 1, MW standards; lane 2, trypsin; lane 3, undigested PfCRT; lane 4, PfCRT digested with trypsin for 2.5 min.

The labeled protein fragment was excised from the silver stained gel (Figure 2A) and sent for mass spectrometry analysis. The peaks from the resulting mass spectrum (Figure 3) were examined for their amino acid composition. A few peaks corresponded to keratin (a ubiquitous contaminant from human skin) and one to a trypsin autolytic fragment. Peptides corresponding to PfCRT amino acids 285 -317 and 405 -424 were also identified. Additionally, one low -mass peak could represent either a tripeptide from keratin or PfCRT amino acids 372-374. Since their masses corresponded exactly to the observed MS peaks +/ - 0.5 Da, these fragments cannot contain the AzBCQ tag.


Final - Shots 1000 - Jenner; Run #2; Label D5
207.045(R5124,S1177)
204.032(R6982,S117)
244.233(R6857,S430)
251.061(R6784,S67)
288.266(R4831,S1591)
286.248(R7625,S198)
317.066(R7316,S593)
323.024(R8321,S19)
365.103(R7571,S227)
379.072(R5826,S1902)
401.056(R8188,S93)
1.8E+4
Mass (m/z)
Final - Shots 1000 - Jenner; Run #2; Label D5
423.115(R7846,S260)
439.110(R8269,S162)
445.017(R9085,S101)
425.121(R6144,S34)
504.109(R7960,S46)
568.117(R9107,S101)
584.161(R7969,S20)
650.026(R10597,S95)
728.535(R6812,S15)
2663.9
Mass (m/z)
Final - Shots 1000 - Jenner; Run #2; Label D5
845.084(R11238,S102)
861.060(R8784,S56)
823.091(R9537,S35)
904.647(R12129,S11)
1066.062(R14387,S42)
1082.055(R8622,S13)
482.4
Mass (m/z)
Final - Shots 1000 - Jenner; Run #2; Label D5
1277.094(R10078,S24)
1247.159(R7963,S13)
1283.080(R20409,S10)
1513.703(R22383,S11)
1591.819(R14734,S17)
76.1
Mass (m/z)
Final - Shots 1000 - Jenner; Run #2; Label D5
1750.849(R22658,S19)
2024.640(R23526,S20)
2005.408(R11472,S11)
2211.088(R10303,S26)
2236.074(R24423,S11)
50
Mass (m/z)
Final - Shots 1000 - Jenner; Run #2; Label D5
2504.629(R10228,S12)
2681.346(R12770,S10)
50
Mass (m/z)
Final - Shots 1000 - Jenner; Run #2; Label D5
3521.005(R33934,S12)
50
Mass (m/z)

mass (Da)	amino acid position	peptide sequence
1768.9380	271-284	QLHLPYNEIWTNIK
1097.5560	285-294	NGFACLFLGR
1335.6395	295-307	NTVVENCGLGMAK
1125.4339	308-317	LCDDCDGAWK
2585.2996	318-339	TFALFSFFNICDNLITSYII DK
2688.3452	340-363	FSTMTYTIVSCIQGPAIAIA YYFK
876.4937	364-371	FLAGDVVR
401.2143	372-374	EPR
2137.1732	375-392	LLDFVTLFGYLFGSIIYR
913.5465	393-400	VGNIILER
147.1128	401-401	K
147.1128	402-402	K
306.1594	403-404	MR
2235.9735	405-424	NEENEDSEGELTNVDSIITQ

Table 1 Predicted tryptic fragments of the C-terminal 17 kDa of PfCRT.

Upon UV activation, the AzBCQ molecule loses N_2, resulting in an adduct with MW 766.25 Da. Subtraction of this value from the masses of all peaks displayed in the peptide m/z spectrum yielded only one peak that corresponds to a predicted tryptic fragment residing with the C-terminal region of PfCRT. That peak has m/z of 2024.64, which is within 0.30 Da of that expected for the peptide defined by residues 364 – 374 (Figure 4, bold). The AzBCQ must therefore be attached to one of these amino acids. Moreover, the last three amino acids of this segment are those that may correspond to the low-mass unlabeled fragment mentioned earlier, thereby further narrowing the possible positions for the label. These residues are believed to comprise the intra-digestive vacuolar loop connecting putative helices 9 and 10 (Figure 4, underlined) of PfCRT protein, and contained within this sequence is position 371, which is known to be mutated in some CQ-resistant strains.

…QLHLPYNEIWTNI*K**NGFACLFLGR**NTVVENCGLGMAK*
*LCDDCDGAWK*TFALFSFFNICDNLITSYIIDKFSTMTY
TIVSCIQGPAIAIAYYFK**FLAGDVVREPR**LLDFVTLFG
YLFGSIIYRVGNIILERKKMR*NEENEDSEGELTNVDSI*
ITQ

Figure 4 *C-terminal sequence of PfCRT containing the AzBCQ binding site.* Shown are amino acids 284-324, corresponding to the last 18.6 kDa of the protein. Unlabeled peptide fragments identified by MS are in *italics*, predicted transmembrane domains are underlined, and the AzBCQ-bound fragment is in **bold**.

Finally, I collaborated with a protein modeler to elucidate the nature of the CQ-PfCRT interaction in 3-dimentional space. Previous work with drug-selected parasite lines [8] suggested that helices 1 and 9 are important to the protein's drug-interaction capabilities, and energy-minimized models are consistent with this hypothesis (Figure 5).

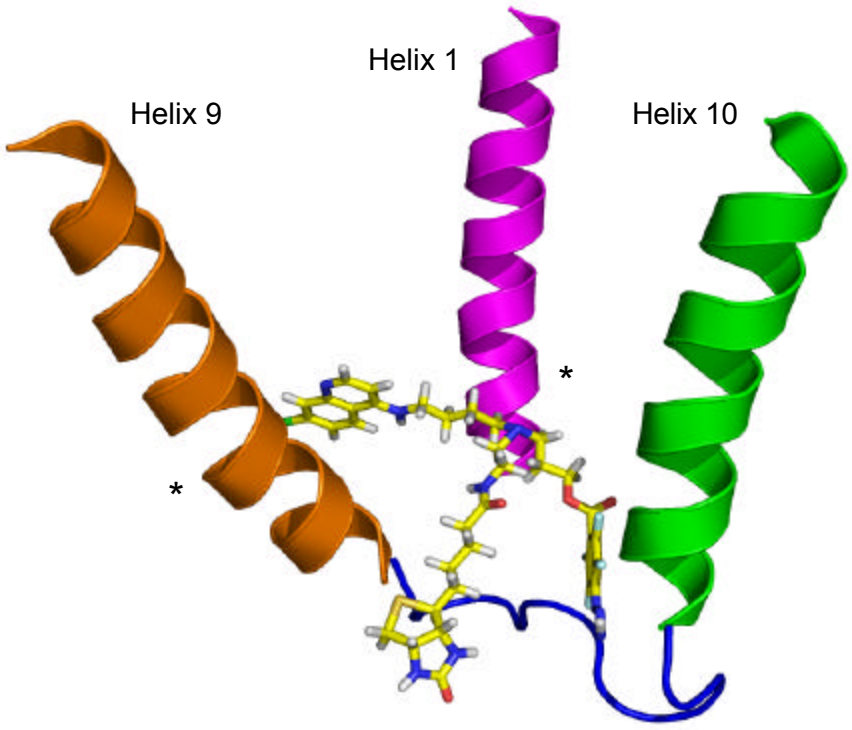


Figure 5 *Ribbon diagram of proposed PfCRT drug binding pocket*. The CQ pharmacophore is predicted to lie within the lower third of the membrane, while the bulky biotin and perfluorophenyl azido groups stretch into the parasite digestive vacuole, with the latter making contact with (and inserting into) the interhelix loop of amino acids. Asterisks mark sections of helices that contain mutations associated with CQ resistance.

KEY ACCOMPLISHMENTS

Research Accomplishments

- Definition of the PfCRT CQ-binding site

Training Accomplishments

- Successful defense of dissertation, June 30 2008
- Graduation with Doctorate in Tumor Biology

REPORTABLE OUTCOMES

- Published Thesis: Lekostaj, JK. (2008) Molecular Analysis of Membrane Transporters Implicated in Drug Resistance.
- Published Article: Lekostaj JK, Natarajan JK, Paguio MF, Wolf C, Roepe PD. (2008) Photoaffinity labeling of the *Plasmodium falciparum* chloroquine resistance transporter with a novel perfluorophenylazido chloroquine. *Biochemistry*. 47(39): 10394-406.

CONCLUSION

In the three months of this year supported by this research grant, I utilized photoaffinity labeling combined with enzymatic digestion and mass spectrometry to define the drug binding site of PfCRT, a member of a drug/metabolite transporter superfamily that is thought to be vitally important in malarial multidrug resistance. My data supports a direct physical interaction between the protein and the drug, which allows for the possibility of active drug efflux, or pumping. Furthermore, the identified drug binding pocket is conceptually consistent with data from other investigators that together offer potential explanations for the differences between wildtype and mutant proteins.

A request for a change of PI for this award has been submitted and is pending review of this summary.

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APPENDICES

none